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«Analysis of the phase variation rate of the *swrA* gene in *Bacillus subtilis*»

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΤΗΣ ΠΑΒΙΑΣ

Τμήμα Βιολογίας και Βιοτεχνολογίας

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Τμήμα Βιοχημείας και

Βιοτεχνολογίας

«Ανάλυση του ρυθμού ποικιλότητας φάσης του γονιδίου *swrA στο Bacillus subtilis*»

Supervisor:
Prof. Calvio Cinzia

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Ακαδημαϊκό έτος 2015/2016

ABSTRACT

In *Bacillus subtilis* a protein called SwrA is essential for the expression of various phenotypes like motility and poly-γ-glutamic acid biosynthesis. This protein contains a slippery eight-adenine stretch in the coding sequence that can easily mutate to a non-functional *swrA*$^{-}$ allele that contains nine adenines because of misalignment during replication. Through the same mechanism it can also revert back with a frequency higher than a normal mutation rate (10^{-4}). Thus, the *swrA* gene is regulated through phase variation.

Our laboratory created a strain that contained a specific point mutation, inside the *swrA* coding sequence but outside the poly-$(A)_8$ slippery tract, which presented full motility and polymer production as a wild type *Bacillus subtilis* strain. Unexpectedly, while the wild type strain normally loses the ability to produce γ-PGA after long cultivation because of variation to the *swrA*$^{-}$ phase, the mutant strain maintains productivity. This finding implies that the specific point mutation either produces a SwrA protein with improved functionalities or prevents phase variation by stabilizing the *swrA*$^{+}$ allele. In order to investigate this finding we analyzed the phase variation of this mutant strain in comparison with that of a wild type strain. Finally we found that at lower temperatures this mutation leads to a non-functional or not present protein, evidence that has to be further investigated and analyzed on the future

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ΠΕΡΙΛΗΨΗ

Στο *Bacillus subtilis* μια πρωτεΐνη που ονομάζεται SwrA είναι σημαντική για την έκφραση διαφόρων φαινοτύπων όπως η κινητικότητα και η βιοσύνθεση του γ-PGA. Η πρωτεΐνη αυτή περιέχει μια περιοχή από 8 συνεχόμενες αδενίνες στην κωδική της αλληλουχία, η οποία μπορεί εύκολα να μεταλλαχθεί στο μη λειτουργικό αλληλόμορφο το οποίο περιέχει την ίδια περιοχή με την προσθήκη όμως μιας ένατης αδενίνης λόγω λάθους κατά την διαδικασία της αντιγραφής. Με τον ίδιο μηχανισμό μπορεί να επιστρέψει στην αρχική κατάσταση με συχνότητα υψηλότερη της φυσιολογικής μετάλλαξης (10^{-4}). Επομένως, το γονίδιο *swrA* ρυθμίζεται μέσω του μηχανισμού της ποικιλότητας φάσης.

Το εργαστήριο μας δημιούργησε ένα βακτηριακό στέλεχος το οποίο περιείχε μια συγκεκριμένη μετάλλαξη σημείου, μέσα στην κωδική αλληλουχία του *swrA* γονιδίου αλλά εκτός της πολυαδενυλικής αλληλουχίας, η οποία παρουσίασε πλήρη κινητικότητα καθώς και σύνθεση του πολυμερούς όπως και το άγριου τύπου βακτηριακό στέλεχος του *Bacillus subtilis.* Απρόσμενα, ενώ το στέλεχος άγριου τύπου φυσιολογικά χάνει την ικανότητα να παράγει το γ-PGA μετά από μεγάλη σε διάρκεια καλλιέργεια λόγω της μεταβολής φάσης σε *swrA*$^{-}$, το μεταλλαγμένο στέλεχος την διατηρεί. Το εύρημα αυτό υπονοεί πως η συγκεκριμένη μετάλλαξη σημείου είτε παράγει μια swrA πρωτεΐνη με βελτιωμένες λειτουργίες είτε αποτρέπει την μεταβολή φάσης σταθεροποιώντας το φυσιολογικό αλληλόμορφο *swrA*$^{+}$. Για να ερευνήσουμε το εύρημα αυτό, αναλύσαμε τον ρυθμό μεταβολής φάσης του μεταλλαγμένου στελέχους σε σύγκριση με αυτό του άγριου τύπου. Τελικά,

βρήκαμε πως σε χαμηλότερες θερμοκρασίες η μετάλλαξη αυτή οδηγεί σε μια μη λειτουργική ή απούσα πρωτεΐνη, στοιχείο που πρέπει να ερευνηθεί και αναλυθεί περαιτέρω στο μέλλον.

Η παρούσα διπλωματική εργασία εκπονήθηκε στο εργαστήριο Microbial Genetics of the Department of Biology and Biotechnology "L. Spallanzani" , University of Pavia.

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INTRODUCTION

Bacillus subtilis

Bacillus subtilis is a rod-shaped, Gram-positive bacterium that is commonly recovered from soil, water, air, and decomposing plant material. It has been considered strictly aerobic; however, recent studies show that it can also grow in anaerobic conditions making it a facultative aerobe. Probably it is the best studied Gram-positive bacterium and a model organism to study bacterial chromosome replication and cell differentiation.

Stress and starvation are present in the environment; therefore *Bacillus subtilis* has evolved a set of strategies that allow survival under these harsh conditions, like formation of stress-resistant endospores or transformation with external DNA.

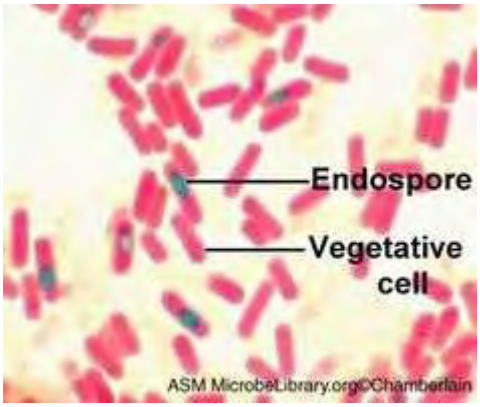


Fig. 1: *Bacillus subtilis* spores stain (Neal Chamberlain, ASM MicrobeLibrary).

Bacillus subtilis has one circular chromosome. The total size of the entire DNA is 4.2 Mbp and 53 % of the protein-coding genes are only present once, while 25 % of the genome relates to families of genes that have undergone gene duplication (Kunst et al., 1997). The majority of essential genes are related to cell metabolism, with about half involved in information processing, one-fifth involved in the synthesis of cell envelope and the determination of cell shape and division and one-tenth related to cell energetics (Kobayashi et al., 2003).

There are a lot of applications that involve *Bacillus subtilis*, mainly in industrial environment, agriculture and medicine. The ability of selected *Bacillus* strains to produce and secrete large quantities (20-25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability to ferment in the acid, neutral and alkaline pH ranges,

combined with the presence of thermophiles in the genus, leads to a variety of new commercial enzyme products with desired characteristics (Schallmey et al., 2004). *Bacillus* strains have also been developed and engineered as industrial producers of nucleotides and poly-gamma-glutamic acid. Together with *Escherichia coli* they create great model systems for a lot of genetic experiments.

Two-component system DegS/DegU

Generally, a two-component regulatory system serves as a basic stimulus-response coupling mechanism regulating many bacterial responses, like the reaction to environmental changes. It consists of a histidine kinase (HK) responsible for sensing an environmental stimulus and a response regulator (RR) who interferes with the cellular response through signaling pathways or genes expression (Fig. 5). These two proteins are able to transmit all kinds of signals by using a phosphorelay (a transfer of a phosphate group from protein to protein) in order to ensure cell survival. Firstly, the HK is able to sense a signal; it binds ATP and auto phosphorylates on a catalytic His residue. Subsequently, the RR is phosphorylated on a catalytic Asp residue via the phosphoryl group transferred from the phosphorylated His residue of the HK. The phosphorylated RR generally dimerizes and interacts with other proteins or DNA to exert a genetic program, while other regulators are alleviated of inhibition by their N-terminal domain. Mainly, these systems exist in prokaryotic organisms while only a few have been identified in eukaryotic organisms (Gross and Beier, 2012) (Fig. 5).

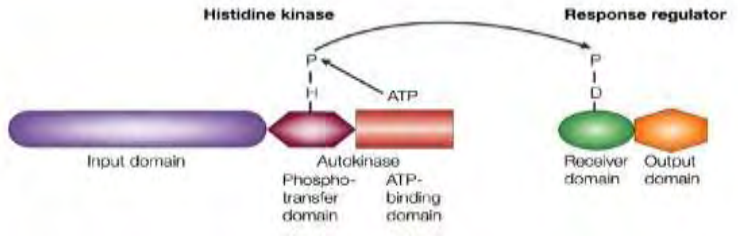


Fig. 2: Interaction between the histidine kinase and the response regulator that creates a two-component regulatory system (Rasmus et al., 2002).

The response regulator DegU and its cognate histidine kinase DegS establish a two-component system in *Bacillus subtilis*. DegU undergoes phosphorylation and dephosphorylation by DegS, which is both kinase and phosphatase. The *degS/degU* genes are transcribed as an operon and *degU* is

also transcribed from two additional internal promoters: one activated by DegU~P itself and the other by nitrogen starvation (Jers et al., 2011). Two additional regulatory peptides, DegQ and DegR, play a significant role in DegS/DegU activity; notably DegQ enhances the phosphorylation of DegU by DegS but does not prevent it from dephosphorylation, while DegR stabilizes the phosphorylated form of DegU (Kobayashi, 2007; Tokunaga et al., 1994).

This turnover between phosphorylation and dephosphorylation of DegU is essential for the immediate cellular response to environmental changes and the numerous processes that characterize the changeover from the exponential to the stationary growth phase, including the induction of several enzymes and the development of genetic competence leading to the conclusion that DegU plays crucial role in cell fate. The phosphorylated DegU activates a lot of processes like production of degradative enzymes, synthesis of γ-PGA and motility which we are going to analyze below (Tsukahara and Ogura, 2008).

Scientists isolated specific mutations in DegS and DegU, $degS_{200}Hy$ and $degU_{32}Hy$ respectively, because of their ability to increase exoproteases production. These mutations stabilize the phosphorylated form of DegU increasing the protein function, as stated in the last paragraph. Specifically, $degS_{200}Hy$ is a G218E mutation that inhibits the phosphatase activity of DegS while $degU_{32}Hy$ carries the mutation H12L that stabilizes the phosphorylated form of DegU. However, the $degU_{32}Hy$ mutant proteins has probably additional properties and does not behave as a simple DegU~P (Mordini et al., 2013).

Motility

Bacterial flagella consist of 3 major parts: the filament, a very long protruding structure which acts as a propeller and behaves differently depending on which way the motor turns; the hook, a universal joint which transmits motor torque to the flagellum even if it is curved and an ion driven motor, which can provide a torque in either direction (Arkhipov et al., 2006; Patrick and Kearns, 2012). Flagella are necessary for both swarming and swimming motility. Swarming takes place in solid and semi-solid surfaces and requires an increase in flagellar biosynthesis, cell-cell interactions, and also the presence of a bacterially produced surfactant (surfactin for *B. subtilis*). Swarming is promoted by high growth rates and requires energy-rich conditions. Swimming on the other hand is the typical motility in liquid media operated by single oligoflagellated cells (Cozy and Kearns, 2010) (Fig. 6). Media solidified with agar concentrations above 0.3 % exclude swimming motility and force

bacteria to move, if possible, by swarming, whereas agar concentrations above 1% prohibit swarming of many bacterial species.

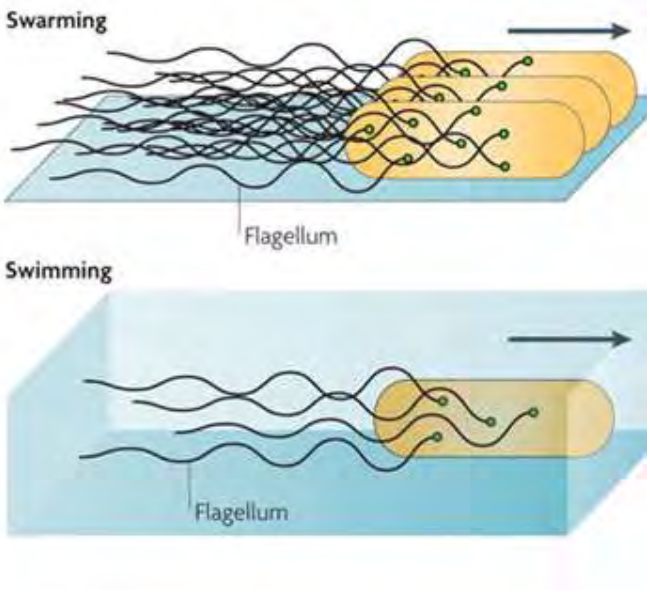


Fig. 3: Swarming is the multicellular movement of bacteria across a surface and is powered by rotating helical flagella while swimming is the movement of individual bacteria in liquid, also powered by rotating flagella (Kearns, 2010).

Most of the flagellum components are encoded by an operon called *fla/che* operon. This operon contains also the gene for the alternative sigma factor SigD which is necessary for the transcription of the flagellin gene (Cozy and Kearns, 2010). SigD is also needed for the expression of the motor proteins and autolysins (enzymes involved in cell-cell separation by breaking down the peptidoglycan). The *fla/che* operon and its role in motility are explained in detail below.

SwrA

Differently from wild strains, domesticated laboratory strains of *Bacillus subtilis* are not able to swarm, but they maintain their ability to swim. This observation triggered a series of experiments that led to the discovery of 2 different mutations present in laboratory strains that impair their ability to swarm: one is in the gene responsible for surfactin synthesis and the other in a gene named *swrA*. The wild type *swrA* sequence contains an 8 A:T base pairs stretch in the coding portion while in the laboratory strains one extra A:T base pair is inserted leading to a frame-shift that inactivates the gene product (Kearns et al., 2004). As mentioned above, the *swrA* mutation is due to a nucleotide insertion that prematurely interrupts its reading frame but, occurring in a

polynucleotide stretch, it can easily shift back and restore the protein's functionality. Two promoters regulate *swrA* transcription: a σ^D-dependent promoter $P_{D(swrA)}$ (through a positive autoregulatory mechanism explained below) and a σ^A-dependent promoter that does not play a significant role. Specifically, on solid media both promoters are active and functional while on liquid media only the σ^D-dependent promoter regulates *swrA* transcription. Experiments showed that deletion of either promoter does not inhibit swarming indicating that both promoters are functional even on their own, but the deletion of both leads to a non-swarming phenotype (Calvio et al., 2008).

Fla/che operon

The *fla/che* operon contains 31 genes involved in motility with *sigD* being the most important among them. The operon transcription is impelled by two main promoters: $P_{A(fla/che)}$ and $P_{D3(fla/che)}$. Specifically, the σ^A -dependent $P_{A(fla/che)}$ is the main promoter in *fla/che* expression while the σ^D -dependent $P_{D3(fla/che)}$ is weaker and unable to induce motility alone, because it depends on a gene, *sigD*, encoded inside the operon itself. SigD produced by the activation of $P_{A(fla/che)}$ leads to the activation of $P_{D3(fla/che)}$. This latter promoter thus sustains production of flagella through a weak autoregulatory loop (Mordini et al., 2013).

The master regulator DegU regulates the σ^A-dependent $P_{A(fla/che)}$. Specifically, DegU shows higher affinity to $P_{A(fla/che)}$ when is phosphorylated. In this interaction DegU~P is acting like a repressor of $P_{A(fla/che)}$, inhibiting the expression of the *fla/che* operon (Amati et al., 2004). However, when SwrA is present, it forms a complex with DegU~P at $P_{A(fla/che)}$ and transforms it into an enhancer of *fla/che* transcription. Such enhancement is absolutely required for swarming, which depends on hyper flagellated cells. SigD is thus more expressed, leading to further activation of $P_{D(swrA)}$ and an increase in the expression of SwrA, through an autoregulatory loop (Mordini et al., 2013) (Fig. 7).

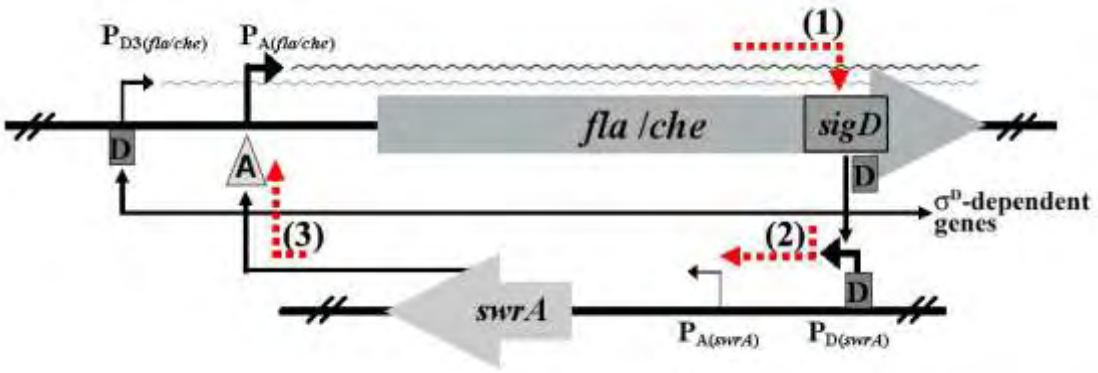


Fig. 4: In *swrA*$^+$ strains $P_{A(fla/che)}$ induces production of SigD which activates *swrA* transcription by binding to $P_{D(swrA)}$. SwrA, in turn, enhances transcription of the *fla/che* operon by binding to DegU~P at the $P_{A(fla/che)}$ completing the autoregulatory loop. SigD also binds to $P_{D3(fla/che)}$ leading to the establishment of a weaker autoregulatory loop that does not include *swrA* at all (Mordini et al., 2013).

Laboratory strains are *swrA*$^-$ but it was observed that the mutation rate towards the *swrA*$^+$ allele is 1/10.000 cells (10^{-4}), a frequency that is comparable to that of phase variation phenomena. Phase variation is defined as the random switching of phenotype (and genotype) at frequencies that are much higher than classical mutation rates (van der Woude and Bäumler, 2004). Indeed, *swrA* is characterized by intense phase variation that causes the presence of a functional (*swrA*$^+$) and a non-functional allele (*swrA*$^-$) simultaneously in a *Bacillus subtilis* culture. This characteristic allows the bacterial population to be constituted by non-motile (*swrA*$^-$) and motile (*swrA*$^+$) cells.

γ-PGA

Besides motility there is another one function regulated by the interaction between SwrA and DegU: γ-PGA production.

Poly-γ-glutamate is an extracellular anionic polymer synthesized by several Gram-positive bacteria members of the Bacillales order, belonging to the *Bacilli* class. Soil bacteria release γ-PGA to increase their resistance on changing environments and also use it as a barrier against phage infections, as a source of glutamate or to survive to high salt concentrations. It can be composed of only L, only D or both glutamate enantiomers forming PDGA, PLGA or PLDGA filaments and it is soluble in water and resistant to proteases, because of the γ-amide linkages between its residues. When pH is less than 7, γ-PGA obtains a structure based on α-helices when at higher pH a β-sheet

based structure. The organism producing the molecule can affect the final size of γ-PGA filaments (Candela and Fouet, 2006).

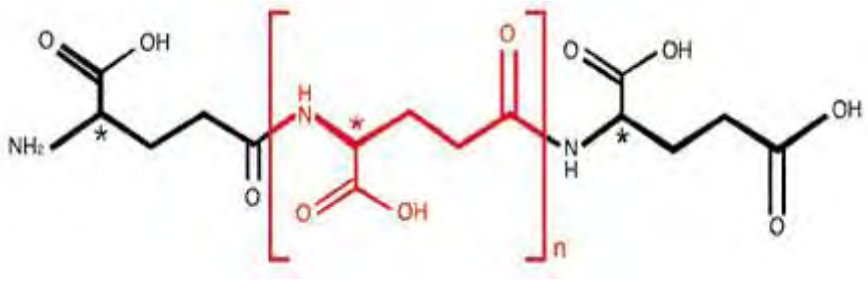


Fig. 5: The chemical structure of γ-PGA.

In *Bacillus subtilis*, four genes (*pgsB*, *pgsC, pgsAA* and *pgsE*), which belong to the *pgs* operon, are sufficient for γ-PGA synthesis (Fig. 9). The *pgs* operon is followed by the *pgdS* gene that codes for a γ-PGA hydrolase (Candela and Fouet, 2006; Osera et al., 2009).

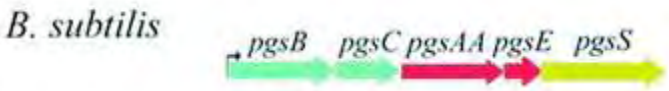


Fig. 6: The *pgs* operon followed by *pgdS* [not *pgsS*] in *Bacillus subtilis* (Candela and Fouet, 2006).

The majority of laboratory strains don't produce γ-PGA because the *pgs* operon is not transcribed. They can be transformed in γ-PGA producers by transformation with both the wild-type $swrA^+$ allele and the $degQ_H$ mutation, a single base substitution in the *degQ* promoter enhancing its expression. DegQ overexpression causes an increase in the level of DegU~P; in fact the same phenotype given by the $degQ_H$ mutation can be obtained with the mutations $degU_{32}$Hy and $degS_{200}$Hy always together with the $swrA^+$ allele. Specifically, in the absence of SwrA, $degU_{32}Hy$ is not capable of inducing γ-PGA production and, in the same way, in the absence of $degU_{32}Hy$ the polymer is not produced by $swrA^+$ strains (Osera et al., 2009).

As stated above, there is a proved cooperation between SwrA and DegU~P in motility and it was demonstrated that these two proteins work together to induce γ-PGA production independently from the activation of *fla/che* operon (Osera et al., 2009).

Mutations in *swrA* promoters have different outcomes in γ-PGA production. When $P_{(swrA)A}$ is mutated there is just a slight negative effect but a mutation in $P_{(swrA)D}$ has a critical effect on *pgs* expression. When both promoters are mutated, a total inhibition of γ-PGA production is observed (Osera et al., 2009).

Phase variation

Phase variation is a mechanism that allows the reversible switch between two or more genotypes/phenotypes. Phase variation is a result of DNA rearrangement that occurs during replication and is inheritable, but can also switch back in the next generations. This literally means that the majority of daughter cells will retain the expression phase of the parent but a minority will have switched expression phase. The phenotype caused by the rearrangement is thus reversible among generations, and the frequency of this reversion exceeds that of a random mutation. Phase variation is often found in pathogens where it is used for regulating the expression of major phenotypes involved in virulence such as flagella production and capsule synthesis (van der Woude and Bäumler, 2004).

SwrA protein contains a slippery 8 A:T base pair stretch in its coding sequence that can mutate and revert back to wild type with a 10^{-4} frequency thus recalling the main characteristics of phase variation. Furthermore, SwrA by switching between a functional $swrA^{+}$ allele and a non-functional $swrA^{-}$ allele regulates motility and γ-PGA production (capsule synthesis) as a typical gene regulated through phase variation (van der Woude and Bäumler, 2004).

SwrA site-specific mutants

In previous work of our laboratory, the sequence of SwrA has been carefully analyzed without finding any relevant similarity to proteins or domains present in database. Therefore, point mutations were inserted in some specific amino acids randomly selected in the coding sequence outside the slippery tract. *Bacillus subtilis* strains carrying the mutant SwrA proteins were compared to a wild-type strain as far as production of γ-PGA and motility were concerned. These SwrA mutants were tested for swimming and swarming ability and showed full motility similarly to the

positive control (*swrA*$^{+}$). Also γ-PGA production was verified in the mutant strains, after the insertion of the *degS*$_{200}$*Hy* mutation in the strains, by the smoother appearance of mutant colonies on LB broth plates. All mutants maintained the ability to induce polymer synthesis. Thus, the mutations introduced did not affect protein function.

However, it was observed that upon long incubation in γ-PGA-producing medium, while normally strains lose the ability to produce the polymer because of mutations in the slippery A:T tract in *swrA*, one of these mutants, strain PB5616, produced high amounts of γ-PGA, even higher than the control *swrA*$^{+}$ strain leading to the conclusion that the specific mutation present in PB5616 either stabilizes the *swrA*$^{+}$ allele, preventing phase variation, or creates a strain with improved SwrA functionalities. The mutation in PB5616 is a point mutation that leads to an amino acid change in the SwrA protein. The strain might be industrially relevant, so the specific mutation will not be disclosed for protecting patenting options. In the rest of the thesis this mutation will be indicated as XXX.

AIM OF THE WORK

The *swrA* allele carries an 8 adenine stretch can easily mutate through replication slippage, generally acquiring an extra adenine (9 adenines), and revert back with a frequency of 10^{-4}. This type of event occurs frequently in genes with polynucleotide tracts and is often involved in phase variation events associated with pathogenic traits (van der Woude and Bäumler, 2004). In this study, we worked with one of the mutated strains created during the above described experiment, PB5616, carrying an undisclosed amino acid mutation in the *swrA* sequence. Preliminary observations indicate that the undisclosed mutations inserted in the *swrA* coding sequence, and which does not involve the slippery poly-adenine tract, might either increase SwrA functions or reduce the frequency of slippage leading to more stable polymer production.

Our goal was to test the mutation rate of this mutant strain in comparison to a wild type strain. We decided to use γ-PGA production as the criteria to discriminate *swrA*$^{+}$ and *swrA*$^{-}$ colonies being a very simple phenotype due to its distinct appearance and easy display.

MATERIALS AND METHODS

Media

- **Lucia-Bertani medium** (LB)

Tryptone	4 g/l
Yeast Extract	2 g/l
Sodium Chloride	4 g/l

Dissolve components in 400 ml of dH_20 and put them in autoclave at 121^oC for 20 min.

For plates: add Agar (15 g/l) in the mixture and autoclave. When the medium is still warm, add the required antibiotics; pour in plates and store at 4^oC.

- **Nutrient Broth** (NB)

Dissolve 8 g of the medium in one liter of dH_2O; mix and autoclave at 121^oC for 20 minutes. Add the necessary antibiotics when the medium is still warm and pour in plates. Store at 4^oC

- **LM**

LB	10 ml
$MgSO_4$ 1M	30 µl
Nutritional request 5mg/ml	50 µl

Requested nutrients for our strains are Tryptophan and Phenylalanine

- **MD**

PC 1X*	9.2 ml
Glucose 50%	0.4 ml
K-aspartate 100mg/ml	0.25 ml
Nutritional request 5mg/ml	0.1 ml
NH4-ferric citrate 2,2mg/ml	0.05 ml
$MgSO_4$ 1M	0.03 ml

*PC10X:

K_2HPO_4	107 g/l
KH_2PO_4	60 g/l

Na-citrate $5H_2O$	10 g/l
pH 7	

- **SSC 20X** (amount for 1 liter)

NaCl	175.3 g
Na_3Citrate	88.2 g
pH 7	

- **TBAB** (Tryptose Blood Agar Base)

For 400 ml: dissolve 13.2 g of Tryptose Blood agar base medium in 400 ml of dH_2O and autoclave at 121^oC for 20 min. Add the necessary antibiotics when the medium is still warm and pour in plates. Store at 4^oC.

- **PY** (In one liter)

Peptone	10 g
Yeast extract, dehydrated	1 g
$MgSO_4$ $7H_2O$	2 g
$(NH_4)_2SO_4$	2 g
pH 7	
Autoclave at 121^oC for 15 minutes.	
Add Glucose 50% when it is necessary	

- **Swimming medium** (amounts for 400 ml)

Agar (0,2%)	0.8 g
Yeast extract (5g/l)	2 g
Bacto Tryptone (10g/l)	4 g
NaCl (10g/l)	4 g

Dissolve components in 400 ml of dH_2O and autoclave at 121°C for 20 minutes. Pour 25 ml of medium into each plate by using a pipetting aid, applying a rotatory motion to ensure a correct mixing of the components. Plates are ready after 90 minutes.

- **LB or NB Skim milk plates** (1,6 % skim milk)

  Dissolve 16 g of skim milk in 100 ml of dH_2O [16 %]; mix well and autoclave at 121^oC for 20 minutes. Dilute the skim milk 10X in medium (either LB or NB) in order to have final concentration of skim milk 1.6 % on the plate.

- **Davies MM 2X** (amounts for 500ml)

$(NH_4)_2SO_4$	1 g
K_2HPO_4	7 g
KH_2PO_4	2 g
Na_3-citrate	0.5 g
$MgSO_4$	0.1 g
Asparagine	1 g

- **Minimal Media** (MM)

  Prepare 2 different solutions, one with 1.5 % agar in H_2O and the Davies MM2X. Before making plates, add Glucose 50 % of the final concentration in the agar solution. Mix the solutions and add the necessary antibiotics on the plate before pouring the medium. In our case, we used kanamycin [2 μg/ml] and spectinomycin [60 μg/ml]. This medium is used to grow wild type microorganisms or to select for or against recombinants.

- **EDTA 0,5 M**

  1. Weight 93.05 g EDTA
  2. Dissolve in 200 ml d H_2O water
  3. Add NaOH until solution becomes clear and pH is 8
  4. Adjust the volume to 500 ml with dH_2O

- **TBE** (1 liter of 10X stock)

  1. Dissolve 108 g Tris base and 55 g Boric acid in 800 ml distilled water
  2. Add 40 ml 0.5 M EDTA (pH 8.0)
  3. Adjust volume to 1 Liter
  4. Store at room temperature

The EDTA included in the solution binds divalent metal ions and inhibits metal-dependent nucleases.

Table 1: *Bacillus subtilis* strains used in this work

Strain	Genotype
PB5249	*trpC2 pheA1 swrA*$^+$
PB5370	*trpC2 pheA1 SwrA*$^-$

PB5192	*trpC2 pheA1 SwrA$^+$ P_{fliDST}-lacZ; cat*
PB5128	*trpC2 pheA1 SwrA$^+$ P_{hag}-lacZ; cat*
PB5390	*trpC2 pheA1 SwrA$^+$ $degS_{200}$(Hy); spc*
PB5392	*trpC2 pheA1 PswrAWT swrAA$^+$; kan*
PB5451	*trpC2 pheA1 SwrA$^+$ $degU_{32}$(Hy); spc, kan*
PB5606 tranf. PB5391	*trpC2 pheA1 ΔswrA $degS_{200}$Hy; kan, spc*
PB5616	*trpC2 pheA1 $swrA^{XXX}$; kan*
PB5617	*trpC2 pheA1 $swrA^{ZZZ}$; kan*
PB5392 $degS_{200}$(Hy)	*trpC2 pheA1 PswrAWT swrAA$^+$ $degS_{200}$(Hy); kan, spc*
PB5616 $degS_{200}$(Hy)	*trpC2 pheA1 $swrA^{XXX}$ $degS_{200}$(Hy); kan, spc*
PB5617 $degS_{200}$(Hy)	*trpC2 pheA1 $swrA^{ZZZ}$ $degS_{200}$(Hy); kan, Spc*
PB5392-P_{hag} $degS_{200}$Hy	*trpC2 pheA1 P_{swrAWT} swrAA$^+$lacZ $degS_{200}$(Hy); kan, spc*
PB5616-P_{hag} $degS_{200}$Hy	*trpC2 pheA1 $swrA^{XXX}$lacZ $degS_{200}$(Hy); kan, spc*
PB5392-P_{fli} $degS_{200}$Hy	*trpC2 pheA1 P_{swrAWT} swrAA$^+$ lacZ $degS_{200}$(Hy); kan, spc*
PB5616-Pfli $degS_{200}$Hy	*trpC2 pheA1 $swrA^{XXX}$lacZ $degS_{200}$(Hy); kan, spc*

Table 2*:* Primers used in this work

Name	Sequence
Kan-Rev	5’-GTATTTAAAGATACCCCAAGAAGC-3’
A-Rev	5’-TTGTGAACCCCCATTTTCTTTATACAGATAAGCAC-3’
yvzD2	5’-ACGGAATTCTTATCTCTCTTGCGTCAT-3’

Methods

Bacterial strains and growth conditions

Bacillus subtilis strains used in this study with their relevant genotype are listed in Table 1. All of them were grown at 37^{o}C in Lucia-Bertani broth (LB) or Nutrient broth (NB) supplemented with 1.5 % agar unless otherwise stated. When necessary, media were supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (100 μg/ml), kanamycin (2 μg/ml), chloramphenicol (5 μg/ml), and spectinomycin (60 μg/ml).

Genetic techniques

Bacillus subtilis strains were transformed using chromosomal DNA transformation following Frank Kunst protocols.

Kunst method: Bacteria are inoculated in 2 ml of LM broth and incubated at 37^{o}C overnight with shaking. Next day, the OD_{600} is read and the culture diluted in 10 ml of LM in order to have an OD_{600}=0.2; then the culture is incubated at 37^{o}C with shaking till it reaches an OD_{600} =1 (approximately 2 hours). 0.5 ml of the culture is diluted in 10 ml MD and incubated at 37^{o}C with shaking till bacteria reach the stationary phase. After 4 hours, the entire culture is transferred in a sterile tube and centrifuged at 4000 rpm for 8 minutes at room temperature. Part of the surnatant is discarded paying attention not to touch the pellet. The pellet is resuspended in the remaining surnatant and 0.5 ml of competent cells is transferred in a new sterile tube. DNA (0.05-100 ng chromosomal DNA) is added and cells are incubate at 37^{o}C with shaking for 90 minutes. 150-200 μl of transformed cells are plated on TBAB plates with the necessary antibiotics and incubates at 37^{o}C till colonies are visible.

Chromosomal DNA extraction from *Bacillus subtilis*

Bacteria are inoculated in 2.5 ml of PY + 0.5 % glucose (in order to prevent sporulation) and incubation at 37^{o}C with shaking overnight. The culture is centrifuged at maximum speed for 10 minutes, at room temperature and the supernatant is eliminated. Pellet is resuspended in 200 μl SSC 1X and transferred in a 2 ml eppendorf cup. 10 μl lysozyme are added and incubated at 37^{o}C for 20 minutes (Lysozyme: 20 mg/ml in SSC1X) and then 2 μl of RNase are added. Then, 250 μl of 20 mg/ml proteinase K in SSC 1X are added, then 20 μl of SDS 10% and mixed well by inverting the

tubes. The addition of these solutions in order allows the breakage of cell membranes, degradation of nucleases to avoid DNA degradation. The solution is incubated at 60^{o}C till it becomes transparent (approximately 1 hour). 500 µl of phenol (equilibrated with TE buffer, pH 7.8-8.0) are added, mixed well by inverting the tubes and centrifuged for 5 min at 13000 rpm at room temperature. The upper phase is transferred into a new 1.5 ml eppendorf cup and the extraction phase is repeated with chloroform:isoamylic alcohol (24:1) and mixed well by inverting tubes. Solution is centrifuged for 5 minutes at 13000 rpm at room temperature and the aqueous phase is transferred again in a new 1.5 ml eppendorf cup where 2.5 volume of cold absolute ethanol are added and mixed by inversion. These passages allow chromosomal DNA precipitation. The DNA is now and collected using a Pasteur pipette with a heat-sealed end. DNA is rinsed by dipping onto 200 µl 70% ethanol and finally resuspended in 100 µl of 10 mM Tris-HCl, pH 8. DNA is stored at -20^{o}C.

Motility assays

Cells previously grown on LB broth-agar plates containing the necessary antibiotics were seeded onto the center of 8.5 cm swimming plate with a sterile toothpick. Swimming plates consist of LB broth-0.2 % agar and have to be freshly made. The low concentration of agar is used to maintain a semi-liquid form of the medium after pouring. Plates are normally incubated at 30^{o}C but we also incubated the same strains at room temperature in order to observe the difference in their swimming ability. The strain PB5370 (*swrA*$^{-}$) and 5249 (*swrA*$^{+}$) were used as controls.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a technique to amplify a desired DNA sequence, generating thousands to millions of copies when at least the nucleotides that flank it are known. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. A PCR set up usually consist of the following components:

- DNA template which contains the desired DNA sequence to be amplified
- Two primers, short single stranded DNA oligonucleotides that are complementary to the target sequence
- Taq polymerase or another polymerase that is able to synthesize the new complementary strands to the target sequence

- Deoxynucleoside triphosphates (dNTPs), single units being used by DNA polymerase to "build" the new strand
- Buffer solution, that assures the optimum activity and stability of DNA polymerase
- Magnesium ions

Amplification reactions were performed in a final volume of 20 μl containing:

Taq polymerase	1 U/μl	0.5 μl
Primer forward	20 mM	0.4 μl
Primer reverse	20 mM	0.4 μl
dNTPs	10 mM	0.4 μl
DNA template	30-50 ng/μl	1 μl
Buffer solution	10X	2 μl
$MgCl_2$	50 mM	0.8 μl
dH_20		14.5 μl
Total volume		20 μl

Table 3: PCR sample's final volume

Normally, PCR consists of 25-40 cycles with each cycle consisting of 3 different temperature steps. The temperatures used and the length of time depend on a variety of parameters like the enzymes used for DNA synthesis, the melting temperature of oligonucleotides, and the length of the expected product. In the next table, there is a typical PCR program used in a laboratory.

	Phase	Temperature (^oC)	Time (minutes)
1	Initial denaturation	94	5
2	Denaturation	94	0.5
3	Annealing	50-65	0.5
4	Extension	72	1 min/1000 base pair
5	Final extension	72	5

Table 4: PCR program

Initial denaturation: responsible for denaturing the template DNA

Denaturation**:** breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the newly synthesized strands to separate creating single stranded DNA.

Annealing: primers are bound to the single strand template DNA.

Extension: DNA polymerase extends the primer by its polymerase activity.

Final extension: extend the single stranded DNA that possibly remained unfinished.

Steps 2 to 4 are repeated 25-40X.

Agarose gel electrophoresis

Gel electrophoresis is a method of separation and analysis of DNA, based on its charge and according to its size. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose. Shorter molecules move faster and migrate farther than longer ones because they migrate more easily through the pores of the gel. Electrophoresis is performed in buffer solutions (generally TBE1X) in order to reduce pH changes due to the electrical field. The speed of migration depends on some parameters like the percentage of agarose on the gel and the size of molecules. This method is usually performed for analytical purposes, often after amplification of DNA via PCR. There are some molecular weight size markers available that contain a mixture of molecules of known size. If such a marker runs on one lane in the gel parallel to the samples, its bands can be compared to those of our samples in order to determine their size.

In order to prepare an agarose gel, we have to pour 1.5 % (w/v) Agarose powder into microwavable flask along with TBE1X. The agarose is dispersed in the buffer before heating it to near-boiling point, but avoid boiling. The melted agarose is allowed to cool sufficiently in order to add ethidium bromide to a final concentration of 0.2-0.5 μg/ml that binds to DNA, fluoresce under ultraviolet light and allows DNA to be visualized. Before loading our sample, we mix it with Loading Dye 6X which contains a high percentage of glycerol that finally makes the sample heavier than water and will settle to the bottom of the gel well instead of diffusing in the buffer. Run the gel in approximately 135 V and when the dye line is around 80% of the way down to the gel we turn it off and visualize the gel in UV in λ=260 nm. The visible bands correspond to our fragments and for

determining the size and concentration of them we compare the fragment's band with the molecular marker that we loaded in a different gel well.

Purification of PCR products

The PCR purification protocol achieves rapid and efficient removal of short primers, dNTPs, enzymes, short-failed PCR products, and salts from PCR fragments >100 base pair, typically in less than 10 minutes. In our laboratory, we use Micro Clean resin to purify the PCR products. Initially, 1 volume of resin is added in the sample; mixed very well and left at room temperature for 5 minutes. It is centrifuged at room temperature for 5 minutes at 14.000 rpm and the supernatant is then eliminated. The centrifugation is repeated and the possibly remained supernatant is eliminated. The sample is finally resuspended in Tris-HCl.

When we want to send our samples for sequencing, we don't resuspend our DNA. In case our primer is not a common one we have to add it in our sample before drying it at 72^{o}C. We need 6.4 pmoles of primer in 0.5 μl for each sequencing reaction.

Growth curve

The growth curve describes the growing cycle of a bacteria culture. Specifically, the growth of bacteria is divided on four different phases: lag phase, exponential phase, stationary phase and finally death phase. During lag phase, bacteria are maturing and are not yet able to divide while exponential phase is characterized by bacteria doubling. Stationary phase describes a situation in which growth rate and death rate are equal and finally on death phase bacteria die. In order to create a growth curve of our bacteria, OD_{600} is measured every 30 min, using a sterile pipette. Each measurement is recorded and the growth curve is created on Microsoft Excel. Specifically, time is placed on the X axis while OD_{600} measurements are placed on the Y axis on a logarithmic scale.

Strain inoculum

To start from single colonies of similar size, the two strains were inoculated in a sterile tube with 2.5 ml LB and 0.5 μl kanamycin [2 μg/ml] and incubated at 37^{o}C with shaking for 8 hours. Afterwards, $0D_{600}$ was read and the 2 strains were plated separately on LB with appropriate serial dilutions so to have 100 colonies per plate. Next day, for each strain one single colony of similar in size and shape was picked and pre-inoculated in 2.5 ml LB. After four hours, OD_{600} of the pre-

inoculum was read and cultures were inoculated in 38 ml of LB in 2 separate sterile flasks with an initial OD_{600}=0.2.

The growth curve was followed and captured till the stationary phase and the culture was further incubated at 37°C with shaking. During the next days OD_{600} was read approximately every 9 hours and one aliquot from both the strains was plated on 7 LB plates upon serial dilution in order to have 150 cells per plate. Plates were incubated over-night at 37°C and the next day the total number of normal (γ-PGA producing) and mutant (dry) *Bacillus subtilis* colonies could be counted and recorded. This specific experiment was repeated 3 times.

The role of temperature in γ-PGA production

We used two different procedures to verify the role of temperature on γ-PGA production.

1st method: Plates used in this method are LB broth-agar containing four streaks of each strain. Plates are incubated in four different temperature conditions which are:

I.	Room temperature	→	37°C
II.	Room temperature	→	Room temperature
III.	37°C	→	Room temperature
IV.	37°C	→	37°C

Initially, the plates are incubated at the first indicated temperature for 6 hours and when it is necessary (I and III) are transferred to the other one. The final observation is made after 24 hours from the incubation.

2nd method: Strains were tested for their ability to produce γ-PGA on LB broth-agar plates at 3 different temperatures: 37°C, 30°C and 26°C. The same plate consists of 3 streaks from each strain and is incubated at the proper temperature for approximately 24 hours.

Insertion of *pHAG* and *pFLI* genes

In order to transform one some of our strains with the genes *pHAG* and *pFLI*, we followed the transformation's Kunst protocol, as described above. After strain are plated on TBAB plates containing chloramphenicol and kanamycin and incubated at 37°C overnight, colonies producing γ-

PGA are selected because of their mucoid appearance and then tested for degradation of X-gal. Colonies becoming blue are isolated and prepared for extraction of their chromosomal DNA.

RESULTS

Strains construction

As we said before, our goal was to compare the mutation rate between two *Bacillus subtilis* strains. Specifically, the wild type *swrA*$^{+}$ strain (PB5392) and the recently obtained undisclosed mutant PB5616, which carries a mutation in the *swrA* sequence that does not involve the poly-adenine tract and does not impair SwrA functionality. Since γ-PGA production depends on a functional SwrA protein, in order to accomplish this we decided to use it as a convenient phenotype that allows us to observe and count the mutation rate (replication slippage) of our strains by counting the appearance of γ-PGA non-producing strains.

In order to promote γ-PGA synthesis, a *degU/S Hy* mutation is required (Osera et al., 2009). To this end our strains were transformed with the chromosomal DNA of the strain PB5390 containing the *degS*$_{200}$*Hy* mutation linked to a spectinomycin resistance gene. We followed the Kunst transformation protocol, described in Methods. After plating in TBAB plates with spectinomycin and incubating at 37^{o}C overnight, colonies producing γ-PGA were selected because of their mucoid appearance, isolated and prepared for extraction of their chromosomal DNA.

In order to be sure that our transformation was successful and strains maintained the *swrA*$^{+}$ allele we tested the motility of our recombinant strains.

Motility

We tested the motility of our new strains PB5392 *degS*$_{200}$*Hy* and PB5616 *degS*$_{200}$*Hy*. We know that *swrA*$^{+}$ *degS*$_{200}$*Hy* strains should present full motility while the *swrA*$^{-}$ strains should not present motility at all. As control strains we used PB5249 (*swrA*$^{+}$) and PB5370 (*swrA*$^{-}$).

We tested motility of our two strains at 30^{o}C which is the ideal temperature in order to test motility in *Bacillus subtilis*. Motility plates were incubated for 13 hours.

As expected, the control strain PB5249 (*swrA*$^{+}$) presented almost full motility phenotype while the *swrA*$^{-}$ strain presented a reduced motility (because it is not a *degS*$_{200}$*Hy* strain) (Mordini et al., 2013). We repeated the experiment twice. As shown in Fig. 7, both *degS*$_{200}$*Hy* strains PB5392 and

PB5616 presented full motility, confirming the presence of a functional *swrA*$^{+}$ allele. We next proceeded to test the mutation rate of the newly obtained strains.

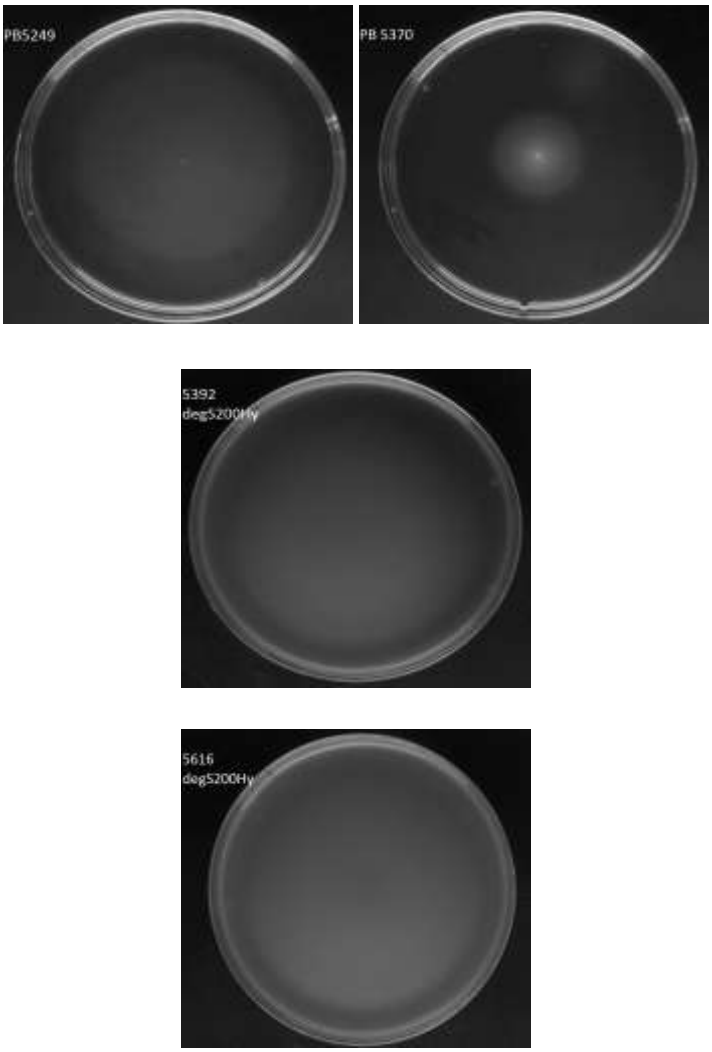


Fig. 7: Motility of the control strains, PB5249 (*swrA*$^{+}$) and PB5370 (*swrA*$^{-}$) are shown on top, left and right, respectively. PB5392 *degS*$_{200}$*Hy* (wild type *swrA*) and PB5616 *degS*$_{200}$*Hy* (*swrA*^{xxx}) are shown below.

Mutation rate

To start from single colonies of similar size, the two *degS*$_{200}$*Hy* strains were inoculated in 2.5 ml LB and incubated at 37^{o}C with shaking for 8 hours. Afterwards, 0D$_{600}$ was read and the strains were plated separately on LB with appropriate serial dilutions so to have 100 colonies per plate. Next day, one single colony similar in size and shape for each strain was picked and pre-inoculated in 2.5 ml LB. After four hours, OD$_{600}$ of the pre-inoculum was read and cultures were used to set a culture with an initial OD$_{600}$ of 0.2 in 40 ml of LB in 2 separate sterile flasks.

The growth curve was followed and captured till the stationary phase (approximately 3.5 hours) and the culture was further incubated at 37^{o}C with shaking for more than 80 hours. A typical growth curve obtained in one of our experiments is shown in Fig. 8. As we can observe, in these conditions bacteria reached the stationary phase after 3 hours of growth.

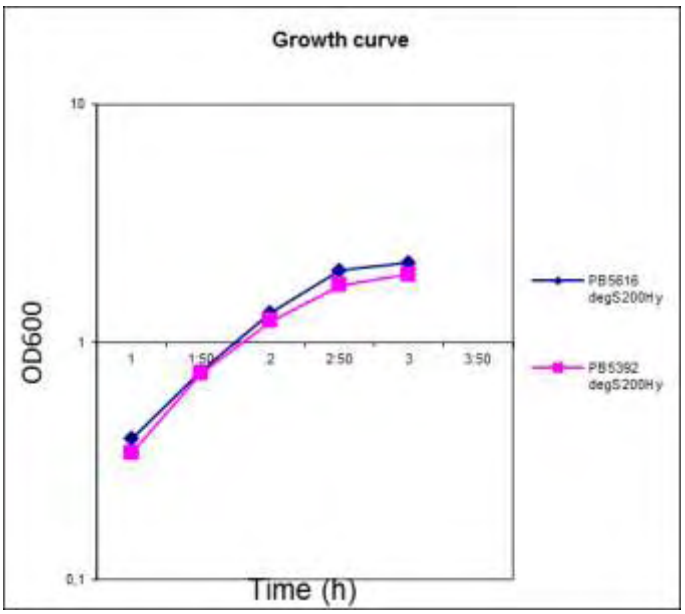


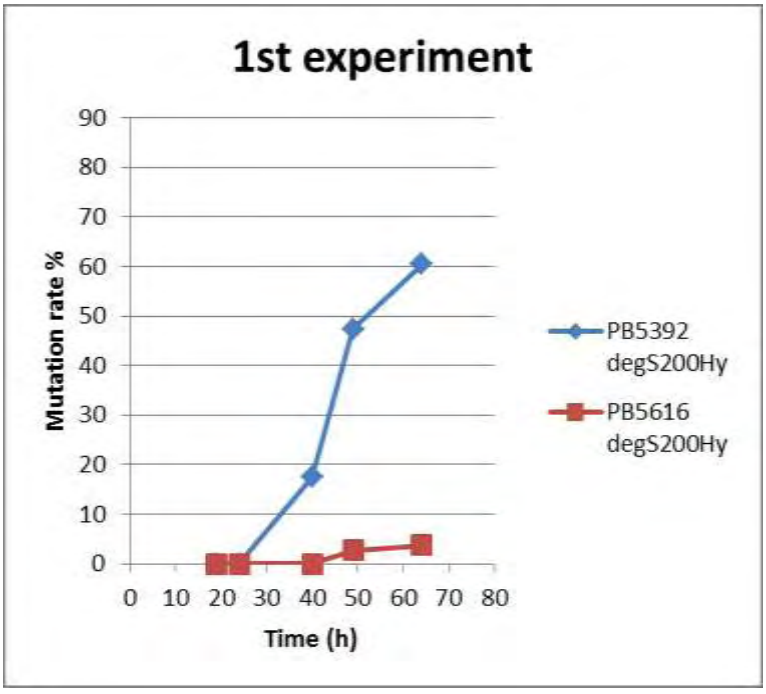
Fig. 8: Growth curve of the two strains.

The OD_{600} was read approximately every 10 hours and from both strains one aliquot was collected and serially diluted in order to have 150 cells per plate ($1.5*10^3$ cells per ml). From the latter dilution seven different LB plates were prepared for each strain at each time point. Plates were incubated overnight at 37°C and the next day the total number of normal (γ-PGA producing) and mutant (flat and dry) *Bacillus subtilis* colonies could be counted and recorded in a Microsoft Excel file. We considered mutant colonies the ones that had a totally flat phenotype. The average number of mutant colonies from the seven plates was divided by the average number of total colonies on the plates and multiplied by 100 in order to obtain the percentage of mutation rate. This specific assay was repeated 3 times and the results are presented in Table 5.

Mutation rate= Mutant colonies/ Total colonies*100

		+19h	+24h	+29h	+40h	+49h	+64h	+73h
1st experiment	PB5392 *degSHy*	0 %		3.51 %	17.5 %	47.4 %	60.49 %	
	PB5616 *degSHy*	0 %		0 %	0 %	2.69 %	3.7 %	
2nd experiment	PB5392 *degSHy*		0 %			6.98 %		85.98 %
	PB5616 *degSHy*		0 %			0.18 %		1.15 %
3rd experiment	PB5392 *degSHy*		0 %		0.76 %	6.13 %	58.71 %	62.65 %
	PB5616 *degSHy*		0 %		0.16 %	0.22 %	1.50 %	1.59 %

Table 5: Mutation rate of the wild type strain PB5392 *degS$_{200}$Hy* and the mutant strain PB5616 *degS$_{200}$Hy*.



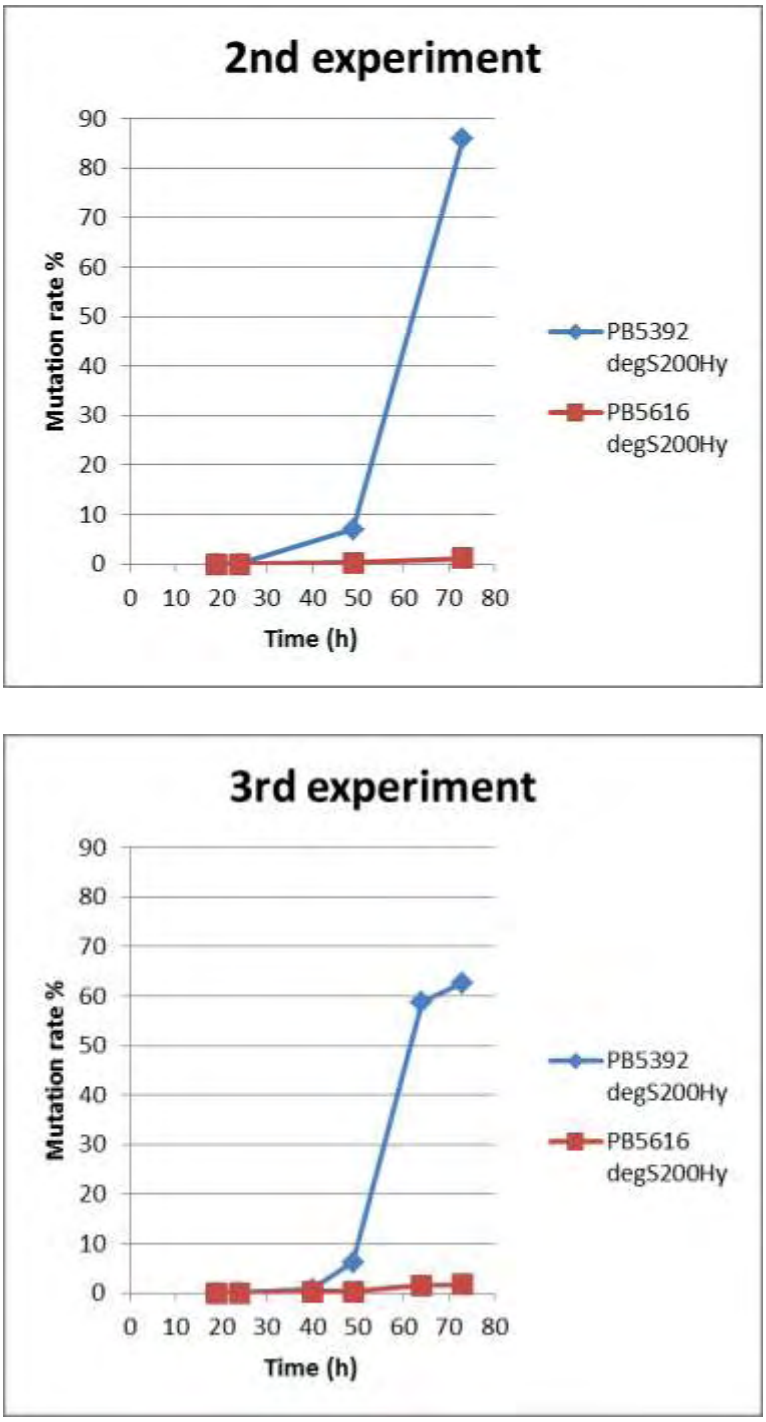


Fig. 9: Mutation rate of the wild type strain PB5392 *degS$_{200}$Hy* and mutant strain PB5616 *degS$_{200}$Hy* in the three experiments carried out.

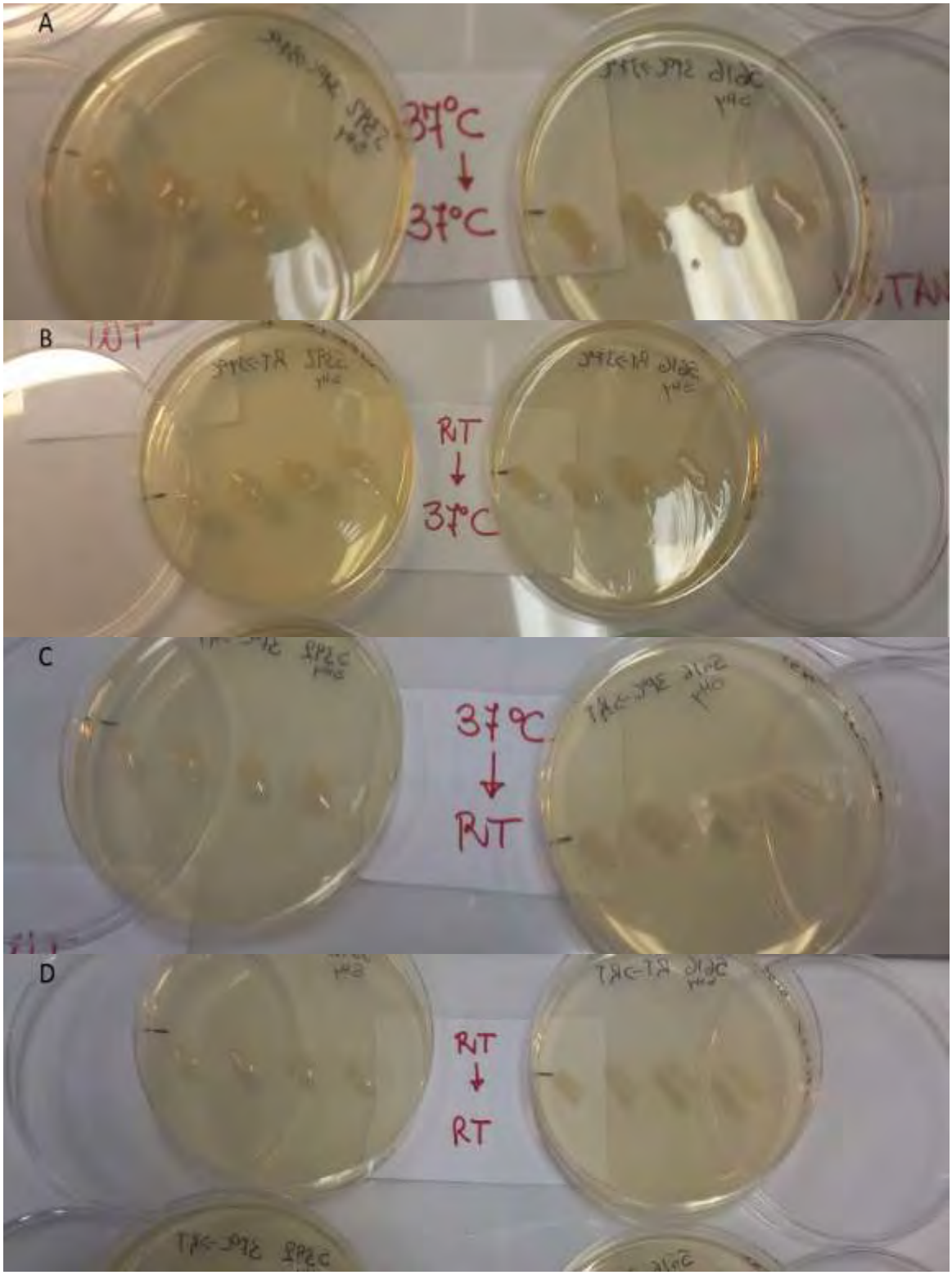
At 24 hours of inoculation both strains were still maintaining their wild-type phenotype, i.e. *Bacillus subtilis* colonies were producing the polymer. Few hours later the mutation rate of the wild type strain PB5392 *degS$_{200}$Hy* increased radically while PB5616 *degS$_{200}$Hy* mostly consisted of γ-PGA producing colonies indicating maintenance of the *swrA*$^{+}$ allele. The mutation rate of the wild type strain was much higher than that of PB5616 in all our experiments, as we clearly observe in Fig. 9.

This result let us conclude that the XXX mutation inserted in the PB5616 strain affects the frequency of variation of the *swrA* gene of *Bacillus subtilis*.

The role of temperature in γ-PGA production

During the incubation of the LB plates in the experiments for verifying the mutation rate we were occasionally forced to use temperatures lower than 37^{o}C, for example for longer incubations over the weekends. This allowed us to observe an unexpected difference on γ-PGA production between the two strains which occurred when they were incubated at room temperature on the laboratory bench. Specifically, we observed that all the colonies of PB5616 *degS$_{200}$Hy* strain did not display the classical mucoid phenotype due to γ-PGA production. This could not be due to *swrA* mutation, as it occurred in all colonies on the plate and in the strain that was not prone to acquire the *swrA*$^{-}$ allele.
To verify this finding we used two different methods: in one case the two strains were streaked in quadruplicate on four LB plates which were then incubated in four different conditions: initially at room temperature and moved at 37^{o}C after 6 hours; initially at 37^{o}C and moved at room temperature after 6 hours; stably at room temperature; stably at 37^{o}C. We took pictures of the plates after a total incubation of 24 hours.
As we can observe from the mucoid phenotype in Fig. 10 A, both strains were producing γ-PGA when incubated stably or mainly (6 hours at room temperature followed by 37°C overnight) at 37°C. As we already knew, incubation at 37^{o}C is ideal for *Bacillus subtilis* colonies to produce the polymer; in fact, colonies grown stably at 37°C were producing more γ-PGA than any other temperature tested.

However, when strains were mainly maintained at room temperature, stably or after a short incubation at 37°C, we observed that their phenotype was drastically different: the strain carrying the wild type *swrA* allele was still presenting the classical mucoid phenotype, although the productivity was reduced in this condition while the *swrA*^{xxx} mutant strain completely lost the ability to produce the biopolymer (Fig. 10 C and D).



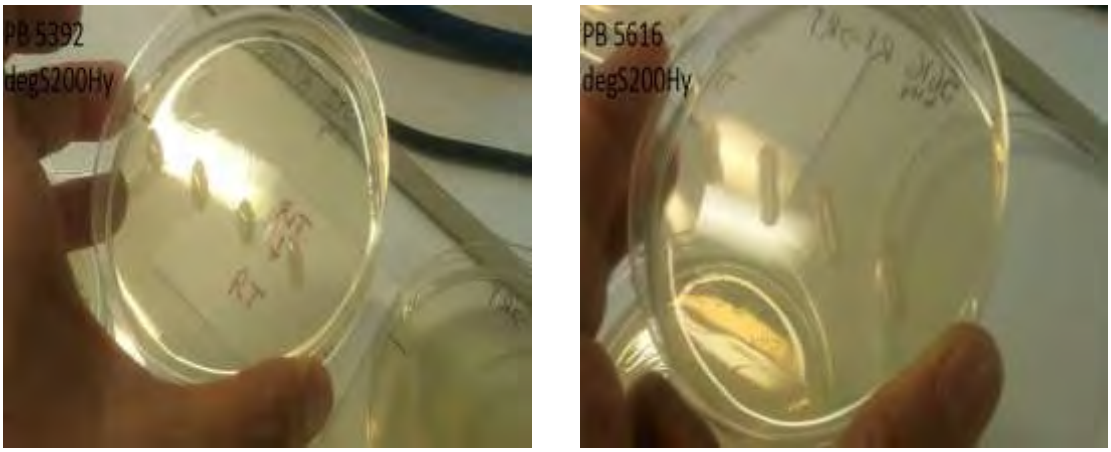


Fig. 10: Plates incubated at four different temperature conditions; the wild-type PB5392 strain is always on the left handside while PB5616 is on the right. On the last two pictures, a detail showing the mucoidy or dryness of the two strains grown at room tempeature.

As the room temperature was not stable for the whole incubation procedure we decided to incubate the plates at more stable and fixed temperatures performing an additional experiment. The two strains were streaked in replicates on three different plates which were incubated for 24 hours at 3 different temperatures: 37^{o}C, 30^{o}C and 26^{o}C.

Both strains were producing γ-PGA when incubated at 37^{o}C although PB5392 *degS$_{200}$Hy* had always a more mucoid phenotype. At 30^{o}C the production of the polymer was reduced in both strains but the reduction was more pronounced in the PB5616 *degS$_{200}$Hy* mutant strain. Finally, at 26^{o}C the wild type strain was still presenting the classical phenotype but with reduced productivity while the mutant strain lost the ability to produce the polymer (data not shown).

These results demonstrated that a higher temperature is necessary for the production and maintenance of γ-PGA production in the mutant strain, although a minor effect can also be observed in the wild type *Bacillus subtilis* colonies. Since the only difference between the strains is in the XXX mutation this implies that the function of the mutant protein or the expression of *swrA* gene carrying this mutation is clearly affected by temperature.

Insertion of *Phag* and *Pfli* genes

In order to analyze the presence of a functional SwrA protein in a more quantitative way, we decided to exploit the effect of SwrA on the expression of *sigD*. SigD is required for the expression of advanced motility genes, such as the flagellin gene *hag* and those contained in the *fli*$_{DST}$ operon.

In the lab, fusions of the promoters of these genes to the *lacZ* gene were already available. These constructs were present in different strains flanked by a chloramphenicol resistance marker. We decided to insert these reporter constructs, constituted by the promoters of *hag* or fli_{DST} fused to the *lacZ* gene, in our strains (PB5392 and PB5616) because these genes are under the control of SigD, and thus also of SwrA. So, we could quantify the activity of the two promoters as an indirect measure of the activity of SwrA. The strains that we used were: PB5392 and PB5616 and they were transformed with 100 ng of chromosomal DNA of the PB5370-*Phag* and PB5370-*Pfli* respectively according to Kunst protocol. Recombinant colonies were selected and tested for the presence of the constructs by degradation of the chromogenic compound X-gal.

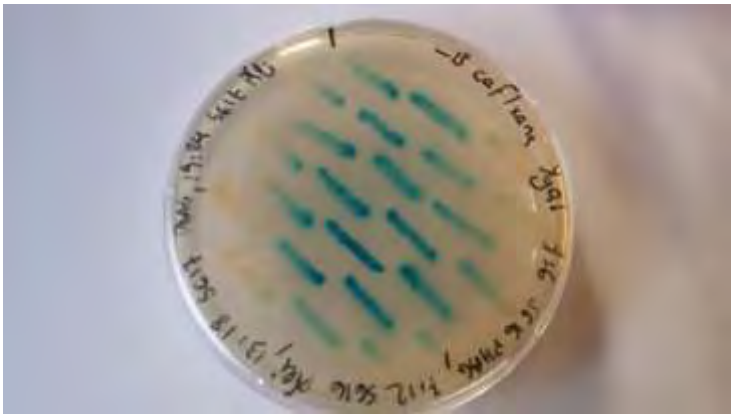


Fig. 11: Blue colonies are the ones that degraded X-gal.

Colonies that degraded X-gal to a greater extent were picked and plated again on LB plates that contained kanamycin. We stored our strains at 4^{o}C. They will be used in future to gain a quantitative measurement of the presence/absence of a functional *swrA* allele.

DISCUSSION

Our experiments indicated that the mutation inserted in the strain PB5616 maintains the *swrA*$^{+}$ allele and decreases the rate of switching to *swrA*$^{-}$ thus leading to the appearance of γ-PGA producing colonies even after long period of cultivation. Thus we can conclude that this mutation inhibits the phase variation of *Bacillus subtilis.* However, the colonies of the mutant strain lost the ability to produce γ-PGA faster than the colonies of the wild type strain after prolonged incubation.

So, to summarize:

- PB5392 *degS*$_{200}$*Hy*: Higher mutation rate but prolonged γ-PGA production for the colonies with normal phenotype.
- PB5616 *degS*$_{200}$*Hy*: Lower mutation rate but production of γ-PGA for a shorter time.

The mechanism that is at the base of this phenomenon is presently unknown. We can hypothesize that the sequence that surrounds the slippery polynucleotide stretch contributes to his propensity to misalign during replication. More experiments are required to prove this hypothesis involving the introduction of specific point mutations in the surrounding region which do not influence the protein sequence (i.e. silent mutations).

Moreover, temperature was shown to play a crucial role in the display of the phenotype. At 37^{o}C both the wild type and the mutant strain produce γ-PGA; however, at lower temperature, while the wild type strain still produces, the mutant strain loses the ability to produce the polymer. Since the only difference between these strains is in the XXX mutation, this finding implies that the SwrA protein produced by the XXX mutant allele is not functional/not present at lower temperature. To explain this evidence we can raise three hypotheses:

1) The protein is not functional at lower temperature because the XXX mutation affects its structure or stability;

2) The SwrA protein is not produced because the messenger RNA cannot be translated

3) The SwrA protein is not produced because the gene is not transcribed

Since it is unlikely that a mutant protein loses functionality at lower temperatures (generally mutant proteins are non-functional at higher temperatures), it is more likely that the coding

sequence of the gene (in which the mutation is localized) plays a role in preventing transcription or translation of the gene itself.

The use of chimeric construct containing a fluorescent gene fused in frame after the *swrA* sequence will allow us to verify expression of the mutant gene at different temperatures. In case expression is absent a northern blot analysis will allow us to discriminate between lack of transcription (no mRNA) and lack of translation.

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